Glycosylation of resveratrol protects it from enzymic oxidation

Gilly REGEV-SHOSHANI*, Oded SHOSEYOV†, Itzhak BILKIS* and Zohar KEREM*[1]

*Institute of Biochemistry, Food Science and Nutrition, The Hebrew University of Jerusalem, P.O. Box 12, Rehovot 76100, Israel, and †Department of Horticulture, The Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, P.O. Box 12, Rehovot 76100, Israel

Plant polyphenols, including dietary polyphenols such as resveratrol, are important components in the plant antioxidant and defence systems. They are also known to exert beneficial effects on human health through diet. As they are produced, these polyphenols may be subjected to deleterious enzymic oxidation by the plant polyphenol oxidases. They are generally synthesized as glycosides like 5,4′-dihydroxystilbene-3-*O*-β-D-glucopyranoside, the 3-glucoside of resveratrol. The effects of the glycosylation and methylation of the parent resveratrol on its enzymic oxidation were studied. Methyl and glucosyl derivatives were synthesized using simple one-step methodologies. The kinetics of their enzymic oxidation by tyrosinases were defined. Substitution at the *p*-hydroxy group, by either glucose or methyl, abolished enzymic oxidation by both mushroom and grape tyrosinases. Substitution at the *m*-hydroxy group with methyl had a small effect, but substitution with glucose resulted in a much lower affinity of the enzymes for the glycoside. We suggest that glycosylation of polyphenols in nature helps to protect these vital molecules from enzymic oxidation, extending their half-life in the cell and maintaining their beneficial antioxidant capacity and biological properties.

Key words: glycoside, oxidation, polyphenol oxidase, resveratrol, tyrosinase.

INTRODUCTION

Polyphenols, including anthocyanins, flavonoids and stilbenes, constitute one of the most abundant and ubiquitous groups of plant metabolites and are an integral part of both human and animal diets. Today, dietary polyphenols are recognized for their beneficial implications in human health, such as in the treatment and prevention of cancer and cardiovascular diseases [1–3]. The wide range of biological effects exhibited by certain polyphenols is generally believed to be the outcome of their powerful antioxidant properties *in vitro*, which are described in numerous publications [2,4,5]. So far, research studies have pertained to cellular systems *ex vivo*, and studies *in vivo* with animal models are scarce [6–9]. Emerging literature, pointing to the low intestinal absorbance of polyphenols, suggests that the ability of polyphenols and their metabolites *in vivo* to interact with cell-signalling cascades, such as apoptosis and redox-sensitive cell-signalling pathways, may be a major mechanism of action [10,11]. In plants, polyphenols are found usually as glycosides or methoxides, or attached to fatty-acid chains. In most naturally occurring flavonols (e.g. quercetin), at least one of the hydroxy groups of the polyphenolic aglycone is glycosylated by mono- or oligosaccharides [1]. Genistein and daidzein, the principal isoflavonoids occurring in legumes, are present primarily as either glycoside conjugates (at position 7) or their 4′-methoxy derivatives [12]. Resveratrol, a naturally occurring hydroxystilbene, may also be found in nature as a glycoside [13] or methoxide [14]. These derivatives differ from the parent aglycones not only in their antioxidant and biological activities, but also in their water solubility and bioavailability [13,15–19]. Interestingly, most polyphenols are not substituted at their ‘reducing’ hydroxy group, i.e. they may still function as antioxidants, but may also be exposed to auto- and pathogenic-oxidizing enzymes [20,21]. It is commonly thought that glycosides exist in nature because of their increased solubility [22] and eventually bioavailability via glucose transporters, but there may be other reasons [23].

In the plant, polyphenols are subjected to deleterious oxidation by the plant's own oxidizing enzymes [24,25]. Moreover, such enzymes, which counteract the biological activities of polyphenols, are also excreted by plant pathogens and exist throughout the digestive system.

Polyphenol oxidases (PPOs) belong to a large family of copper oxidases, which are conserved highly throughout the three kingdoms [26,27]. PPOs are capable of oxidizing a range of phenolic substrates to produce reactive quinones. The role of these enzymes in plant metabolism is not clear, although they may be involved in the plant's defence system [27]. Tyrosinase, a widely distributed plant PPO, is of central importance in vertebrate pigmentation and the browning of fruits and vegetables. This enzyme catalyses the *o*-hydroxylation of monophenols using molecular oxygen (monophenolase activity), and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity). Tyrosinase converts tyrosine into DOPA and then converts DOPA into DOPA-quinone [28–30]. Its active site consists of two copper atoms, which are found in three states: ‘met’, ‘deoxy’ and ‘oxy’. Structural models for the active sites of these three forms have been proposed [28].

Trans-3,5,4′-trihydroxystilbene (*trans*-resveratrol), like other polyphenols, is subjected to deleterious enzymic oxidation by PPOs in plants, during food processing and also after human consumption [24,25,31]. *trans*-Resveratrol is found in grapes and wine, and has attracted special attention due to its therapeutic value. Its health-promoting properties have been reported widely, including oestrogenic and anticarcinogenic effects and protection against cardiovascular diseases [11,32,33]. A few naturally occurring derivatives of *trans*-resveratrol have been identified, in which one or more of the hydroxy groups is substituted. 5,4′-Dihydroxystilbene-3-*O*-β-D-glucopyranoside (piceid) is

Abbreviations used: AUC, area under the curve; BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; piceid, 5,4′-dihydroxystilbene-3-*O*-β-D-glucopyranoside; PPO, polyphenol oxidase; *trans*-resveratrol, *trans*-3,5,4′-trihydroxystilbene.

[1] To whom correspondence should be addressed (e-mail kerem@agri.huji.ac.il).

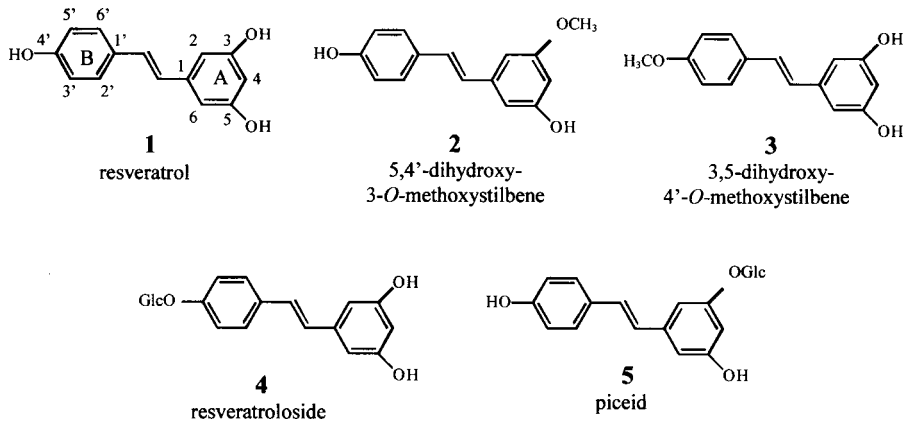

Figure 1 Structure of resveratrol and its derivatives

probably the most abundant form of resveratrol in nature. The number and positions of the hydroxy groups have been suggested to play an important role in its biological activities [18,19,33]. Studying the structural features of polyphenol derivatives in relation to polyphenol-oxidizing enzymes may shed more light on how they are modified in plants and their subsequent function.

In the present study, tyrosinase from mushroom and grape, and resveratrol, served as a model to study how modifications in the polyphenol structure affect parameters of the enzymic oxidation reaction. Glycosides and methoxides of resveratrol (Figure 1) were synthesized and used to study enzyme kinetics.

EXPERIMENTAL

Materials

Tyrosinase, resveratrol, acetobromo-α,D-glucose and potassium hydroxide were purchased from Sigma. Methyl iodide was purchased from Riedel-de Haen (AG Seelze, Hannover, Germany). Ethyl acetate, methanol and potassium carbonate were purchased from Frutarom (Haifa, Israel). Methyl-d_3 alcohol-d was purchased from Aldrich Chemical Company (Milwaukee, WI, U.S.A.). *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Pierce (Rockford, IL, U.S.A.). A cation-exchange column (1 ml HiTrap SP) was purchased from Pharmacia Biotech (Uppsala, Sweden). Carignan grape juice was from Barkan Wineries (Hulda, Israel).

Synthesis of resveratrol methoxides

Resveratrol was methylated by the method of Cardona et al. [34]. Resveratrol (4.38×10^{-5} mol), potassium carbonate (5.04×10^{-5} mol) and methyl iodide (5.04×10^{-5} mol) were dissolved in 1.05 ml acetone. The reaction was stirred overnight at room temperature (20 °C). The next day, 200 μl of double-distilled water was added and acetone was evaporated. Ethyl acetate was used to extract resveratrol and its methylated products from the aqueous phase. The organic phase was evaporated under nitrogen to dryness.

Synthesis of resveratrol glucosides

A modification of the Koenigs and Knorr method for the glycosylation of phenols [35] was developed to glycosylate resveratrol. Resveratrol (10 mg) was mixed with acetobromo-α,D-glucose (18 mg), 24 μl of potassium hydroxide (2.45 mg in ethanol) and 360 μl of ethanol. The mixture was stirred for 1 week at room temperature. Ethanol (500 μl) was then added and HPLC was used to separate the products. For the HPLC analysis, ethanol was evaporated under a nitrogen stream and the dried residue was dissolved in 30 % methanol.

Table 1 Gradient programs for HPLC analyses

Linear gradient programs for elution of methylated and glycosylated products. All compounds were monitored at 306 nm.

Time (min)	Water (%)	Methanol (%)
Methylated products		
0	60	40
2	60	40
5	55	45
10	45	55
23	10	90
25	10	90
25.5	60	40
30	60	40
Glycosylated products		
0	70	30
2	70	30
24	50	50
25	5	95
27	5	95
27.5	70	30
30	70	30

Isolation and purification of stilbenoids

HPLC was used to separate resveratrol and the reaction products of methylation and glycosylation. The HPLC system (Thermo Separation Products, Riviera Beach, FL, U.S.A.) consisted of an auto-sampler (AS3000), injector (100 μl), column oven (35 °C), pump (P3000) and diode-array detector (UV6000). A reverse-phase C_{18} column (250 mm × 4.6 mm; 'Luna' Phenomenex, Torrance, CA, U.S.A.) was employed. Elution was performed using water and methanol, acidified with 0.01 % formic acid, at a flow rate of 1 ml/min. A linear gradient program was developed for each of the reactions (Table 1).

Spectrometry

^{1}H-NMR spectra of the methoxide/glycoside resveratrol molecules were recorded using a Bruker 'Avance' DRX-400

instrument, operating at a frequency of 400.13 MHz for ^{1}H observation. The spectrometer was equipped with a 5 mm Bruker inverse multinuclear resonance probe with a single-axis (z) gradient coil. Spectra were measured at room temperature in $C^2H_3O^2H$. Chemical shifts (p.p.m.) were given on the δ scale; NMR spectra were referenced to internal tetramethylsilane.

GC–MS analyses of the methoxystilbenes were performed with the Varian Saturn-2000 ion trap. A 1 μl aliquot of the sample was injected into the Varian Star 3800 GC. A DB-5 capillary column was used (30 m long, 0.25 mm i.d. and 0.25 μm dry film). The flow rate was 1 ml/min and the injector temperature was 250 °C. The detector temperature was held at 280 °C. The column temperature was set to 80 °C for 1 min, increased to 250 °C at a rate of 20 °C/min, held for 1 min, then increased to 290 °C at a rate of 6 °C/min, held for 2 min and, finally, increased to 300 °C and held for 4 min. The mass spectrometer was operated in Electron Impact mode and electron energy was set to 70 eV. Each methoxystilbene (dried) was treated with 100 μl of BSTFA and heated to 70 °C for 15 min. The reagent was evaporated under nitrogen and dissolved in ethyl acetate as a solvent. Each fraction was diluted 100 times.

Partition coefficients

Partition coefficients were determined according to Privat et al. [36]. Equal volumes of phosphate buffer (50 mM at pH 7.4) and 1-octanol were shaken together and the layers were separated by centrifugation at 1500 ***g*** for 10 min. Resveratrol and its derivatives were each dissolved in 5 ml of buffer-saturated 1-octanol and then 1 ml of the solution was mixed with 1 ml of the buffer solution (in four replicates). The mixtures were shaken for 1 h at 37 °C and then separated by centrifugation. The amount of the compounds in each fraction was measured by HPLC. The partition coefficient was the ratio of the area under the curve (AUC) of 1-octanol solutes to the area of the buffer solution.

Purification of tyrosinase

Tyrosinase was purified from Carignan grapes as described previously [25]. The method included ammonium sulphate precipitation, cation-exchange chromatography, ultrafiltration and gel-filtration chromatography.

PPO activity

PPO activity was defined using DOPA as a substrate. The reaction was performed at 25 °C in 20 mM acetate buffer (pH 6.5) and 6.7 mM DOPA in a total volume of 700 μl. The increase in A_{475} was monitored.

Tyrosinase activity towards resveratrol and its derivatives

The oxidation reaction consisted of 100 μl mushroom tyrosinase (250 units, in acetate buffer), 100 μl of the substrate solution (2.4 mM) and 100 μl of acetate buffer (pH 6.5, 20 mM) containing 0.1 % (w/v) BSA. Substrate solution (100 μl) and acetate buffer (200 μl, pH 6.5) containing 0.1 % BSA served as the control.

Kinetics

K_m values for resveratrol and its methoxide, or glycoside, were determined by a modification of the method as described by Cash et al. [37]. Activity (measured as reduction in the AUC) was measured at 25 °C for various substrate concentrations at pH 6.5. Initial substrate concentrations were 2.4, 1.2, 0.6, 0.3 and 0.15 mM. The substrates (100 μl aliquots) were preincubated with 100 μl of acetate buffer (pH 6.5, 20 mM) containing 0.1 % BSA. The reaction was initiated by adding 100 μl (25 units) of enzyme solution and stopped after 15 min, with 300 μl of acidified methanol. Buffer (100 μl), instead of the enzyme solution, was added as a control. Each sample was tested in four replicates. Reaction products were chromatographed and the AUC determined. K_m and V_{max} values were obtained from a Lineweaver–Burk plot. Statistical analyses of the kinetic parameters were performed using the GraphPad Prism software ($P < 0.05$).

Half-life ($t_{\frac{1}{2}}$) of resveratrol and piceid

In model solution. The $t_{\frac{1}{2}}$ values for resveratrol and piceid were calculated using 10 mM substrate and 150 units of mushroom tyrosinase, using the following equation:

$$t_{\frac{1}{2}} = 0.963/K \quad (K = -\text{slope} \times 2.303) \qquad (1)$$

In grape juice. Fresh Red Glob grapes were pressed and the juice was frozen immediately.

To measure oxidation, the juice (1.8 ml) was mixed with resveratrol or piceid (200 μl, 0.5 mM). The mixture was stirred and aliquots (100 μl) were taken at 5, 10, 15, 20, 30, 60, 120, 180 and 240 min for HPLC analysis. The reaction was performed at 20 °C or at 4 °C, in three replicates each. The $t_{\frac{1}{2}}$ values were calculated using eqn (1).

Quantum chemical calculations

Optimal geometries and heats of formation of resveratrol and its monomethylated and monoglucosylated derivatives were calculated using the restricted Hartree–Fock AM1 Hamiltonian. All calculations were performed with the Spartan 5.1 program package.

RESULTS

Analysis of resveratrol derivatives

An HPLC chromatogram at 306 nm of the methylation products is presented in Figure 2(A). Reaction yields and GC–MS analyses of peaks **1**–**3** (collected from the HPLC), after silylation, are presented in Table 2(A). Compound **1** eluted at R_t 10 min and with UV absorption maxima (280 and 306 nm) identical with authentic *trans*-resveratrol standard. The identity of this compound as resveratrol was further confirmed by GC–MS of the silylated product. It gave a molecular peak at m/z 444, indicating a molecular mass compatible with the molecular formula of tri-(trimethylsilyl)-$C_{14}H_9O_3$. Compounds **2** and **3** had similar absorption spectra ($R_t = 17$ and 18 min respectively). The absorption spectra were identical with resveratrol. GC–MS showed a molecular peak at m/z 386, indicating a molecular mass compatible with the molecular formula of di-(trimethylsilyl)-$C_{15}H_{12}O_3$.

^{1}H-NMR analyses of the purified compounds were performed. The ^{1}H-NMR of peak **2** (Figure 2) is 5,4′-dihydroxy-3-*O*-methoxystilbene: δ 3.80 (s, 3H, OMe), 6.27 (dd, 1 H, $J_{4.2} = J_{4.6} = 2.05$ Hz, H-4), 6.57 (dd, 1 H, $J_{4.2} = J_{4.6} = 2.00$ Hz, H-2 or H-6), 6.58 (d, 1 H, $J_{4.2} = J_{4.6} = 2.00$ Hz, H-2 or H-6), 6.79 (d, 2 H, $J_{3',2'} = J_{5',6'} = 8.40$ Hz, H-3′ and H-5′), 6.87 and 7.03 (AB system, 2 H, $J = 16.28$ Hz, CH = CH), 7.39 (d, 2 H, $J_{2',3'} = J_{6',5'} = 8.77$ Hz, H-2′ and H-6′).

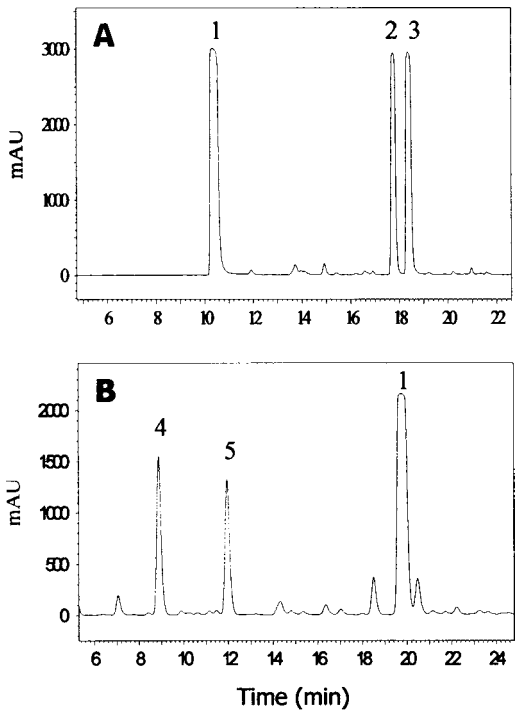

Figure 2 HPLC of the modified resveratrol products

(**A**) Resveratrol methylation products. Elution was performed using water and methanol, acidified with 0.01 % formic acid, at a flow rate of 1 ml/min. A linear gradient program was developed for separating the methylation products (as shown in the Experimental section). (**B**) Resveratrol glycosylation products. Elution was performed using water and methanol, acidified with 0.01 % formic acid, at a flow rate of 1 ml/min. A linear gradient program was developed for separating the glycosylation products (as shown in the Experimental section). The chromatograms were taken at 306 nm.

Table 2 Spectral data of resveratrol derivatives

(**A**) R_t of methylated products obtained from HPLC and from GC after silylation. Molecular ion represents the molecular mass of BSTFA (attached to hydroxy groups) and/or the molecular mass of methyl added to the molecular mass of resveratrol. (**B**) R_t of glycosylated products obtained from HPLC and from GC. Molecular ion represents the molecular mass of glucose added to the molecular mass of resveratrol. Yield is represented as percentage of total products in the reaction.

Peak no.	R_t (min) HPLC	GC	Main molecular ion (*m/z*)	Yield (%)
(**A**) Methylated products				
1	10	14	444	46
2	17	13.69	386	15
3	18	13.13	386	26
(**B**) Glycosylated products				
4	9	–	390	12
5	12	–	390	10
1	19	–	228	70

The ^{1}H-NMR of peak **3** (Figure 2) is 3,5-dihydroxy-4′-*O*-methoxystilbene: δ 3.78 (s, 3 H, OMe), 6.14 (dd, 1 H, $J_{4.2} = J_{4,6} = 2.05$ Hz, H-4), 6.42 (d, 2 H, $J_{2,4} = J_{6,4} = 2.05$ Hz, H-2 and H-6), 6.94 (d, 2 H, $J_{3',2'} = J_{5',6'} = 8.77$ Hz, H-3′ and H-5′), 6.9 and 7.00 (AB system, 2 H, $J = 6.28$ Hz, CH = CH), 7.53 (d, 2 H, $J_{2',3'} = J_{6',5'} = 8.77$ Hz, H-2′ and H-6′).

An HPLC chromatogram at 306 nm of the glycosylation products shows three main peaks (Figure 2B). Reaction yields and

Table 3 Kinetic parameters for the reactions between tyrosinase and resveratrol or its derivatives

Activity of each enzyme (measured as reduction in the AUC) was measured at 25 °C for various substrate concentrations at pH 6.5. Reaction products were chromatographed and the AUC determined. K_m and V_{max} values were obtained from a Lineweaver–Burk plot. Statistical analyses of the kinetic parameters were performed using the GraphPad Prism software ($P < 0.05$).

Substrate	K_m (mM)	V_{max} (μmol/min)	k_{cat} (s^{-1})	k_{cat}/K_m
Mushroom tyrosinase				
Resveratrol	2.0 ± 0.08	4.4 ± 0.2	29.1	14.7
5,4′-Dihydroxy-3-*O*-methoxystilbene	0.7 ± 0.03	3.1 ± 0.1	20.4	28.7
Piceid	8.5 ± 0.5	2.8 ± 0.2	18.8	2.2
Grape tyrosinase				
Resveratrol	1.9 ± 0.1	4.3 ± 0.3	28.4	15.1
5,4′-Dihydroxy-3-*O*-methoxystilbene	0.6 ± 0.01	2.8 ± 0.04	18.6	30.5
Piceid	8.1 ± 0.7	3 ± 0.3	20.0	2.5

MS analyses of peaks **1**, **4** and **5** are summarized in Table 2(B). Compound **1** eluted at R_t 19 min and with UV absorption maxima (280 and 306 nm) identical with authentic *trans*-resveratrol standard. The identity of this compound as resveratrol was confirmed further by MS of the product. It gave a molecular peak at *m/z* 228. Compounds **4** and **5** had similar absorption spectra ($R_t = 9$ and 12 min respectively). The absorption spectra were identical with those for resveratrol. The MS showed molecular peaks at *m/z* 390, indicating monoglycoside resveratrol.

^{1}H-NMR analysis of these peaks was performed. The ^{1}H-NMR of peak **4** (Figure 2) is 3,5-dihydroxystilbene-4′-*O*-β-D-glucopyranoside (resveratroloside): δ 7.47 (d, 2 H, $J = 8.8$ Hz, H-2′, H-6′), 7.01 (d, 1 H, $J = 16$ Hz, H-8), 6.89 (d, 1 H, $J = 16$ Hz, H-7), 7.01 (d, 2 H, $J = 8.8$ Hz, H-3′, H-5′), 6.48 (d, 2 H, $J = 2$ Hz, H-2, H-6), 6.2 (t, 1 H, $J = 2$ Hz, H-4), glucose 4.94 (dd, 1 H, $J = 7.2$, 7.6 Hz, Glc H-1″), 3.92 (dd, 1 H, $J = 11.6$, 2 Hz, H-6″a), 3.7 (dd, 1 H, $J = 11.6$, 5.2 Hz, H-6″b), 3.4–3.56 (m, 4 H, H-2″, H-3″, H-4″, H-5″).

The ^{1}H-NMR of peak **5** (Figure 3) is piceid: δ 7.39 (d, 2 H, $J = 8.6$ Hz, H-2′, H-6′), 7.04 (d, 1 H, $J = 16$ Hz, H-8), 6.86 (d, 1 H, $J = 16$ Hz, H-7), 6.78 (d, 2 H, $J = 8.6$ Hz, H-3′, H-5′), 6.81 (dd, 1 H, $J = 1.4$, 2.1 Hz, H-2), 6.63 (dd, 1 H, $J = 1.4$, 2.1 Hz, H-6), 6.46 (dd, 1 H, $J = 2.1$ Hz, H-4), glucose 4.91 (d, 1 H, $J = 7.2$ Hz, Glc H-1″), 3.95 (dd, 1 H, $J = 12$, 2 Hz, H-6″a), 3.73 (dd, 1 H, $J = 12$, 5.6 Hz, H-6″b), 3.38–3.5 (m, 4 H, H-2″, H-3″, H-4″, H-5″).

Partition coefficients

Partition coefficients were measured for resveratrol and its derivatives and found to be 1.87, 2.51 and 0.89 for resveratrol, dihydroxymonomethoxystilbene and dihydroxystilbene-monoglucopyranoside respectively. The values are represented as the logarithm of (concentration in octanol)/(concentration in buffer) measured by HPLC.

Tyrosinase activity towards resveratrol and its derivatives

Reaction kinetics of mushroom and grape tyrosinases towards resveratrol and its derivatives **2**–**5** were measured. Within 30 min, resveratrol and derivatives **2** and **5** were oxidized completely by the enzyme, whereas the amounts of derivatives **3** and **4** were reduced by only 6–8 %. The affinity and the activity parameters were calculated for resveratrol (peak **1**) and derivatives **2** and **6** (Table 3). The affinity of grape tyrosinase towards resveratrol and its derivatives was higher than that of mushroom tyrosinase in all

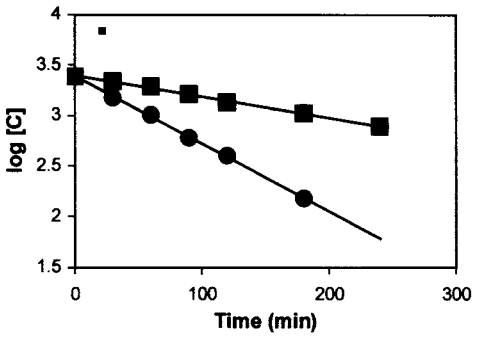

Figure 3 Calculation of $t_{\frac{1}{2}}$ for resveratrol and piceid

Reductions in the amount of resveratrol (●) and piceid (■) during oxidation by mushroom tyrosinase. The results are presented as logarithm of concentrations. r^2 was 0.998 for resveratrol and 0.996 for piceid.

Table 4 $t_{\frac{1}{2}}$ of resveratrol and piceid in grape juice

Reductions in the amount of resveratrol and piceid during 3 h in Red Glob grape juice were measured. The experiment was performed at room temperature (20 °C) or at 4 °C, in three replicates.

Temperature (°C)	$t_{\frac{1}{2}}$ (min)
Resveratrol	
20	6.5 ± 0.6
4	11 ± 0.8
Piceid	
20	36 ± 1.7
4	44 ± 3

cases. Tyrosinase showed a higher affinity for the methoxylated derivatives, and a lower affinity for the glycosylated ones. V_{max} values were independent of the substitution at C-3.

$t_{\frac{1}{2}}$ of resveratrol and piceid

In model solution. To evaluate the effect of the different values of K_m on the oxidation rates of resveratrol and piceid by mushroom tyrosinase, we compared their degradation during 4 h in a reaction mixture (Figure 3). The results were expressed in terms of $t_{\frac{1}{2}}$. The calculated $t_{\frac{1}{2}}$ of piceid was 143 min, which is three times higher than that of resveratrol (45 min), under optimal reaction conditions.

In grape juice. Reductions in the amounts of resveratrol and piceid during 3 h in fresh Red Glob grape juice were measured. The results were expressed as $t_{\frac{1}{2}}$ (Table 4). The calculated $t_{\frac{1}{2}}$, at 20 °C of resveratrol, was 6.5 times lower than that of piceid (36 min). Resveratrol had totally disappeared after 60 min and piceid after 180 min. This difference decreased at 4 °C, with $t_{\frac{1}{2}}$ of 11 min for resveratrol and 44 min for piceid.

DISCUSSION

trans-Resveratrol is a polyphenol unique to red wine, which has attracted much attention due to its high therapeutic value *in vitro*. Simple one-step methods were used in the present study to synthesize glycosylated and methylated derivatives of resveratrol. While resveratrol glycosides, methoxides and polymers are the most abundant stilbenes in nature, their extraction and purification from plants is generally tedious. Only a few studies have demonstrated the glycosylation and methylation of polyphenols using common methods that involve the blocking of hydroxy groups [17,19,38,39]. The difficulties in preparing di- and triphenol derivatives, such as *trans*-resveratrol, arise from the presence of various sites of attack. Moreover, glycosylation can be troublesome with phenols because of the very low reactivity of the phenolic moiety as a glycosyl acceptor, the high pK_a of the aglycone, and the anomerism at C-1 [17,38]. To the best of our knowledge, this is the first report of a one-step glycosylation or methylation of *trans*-resveratrol. HPLC served to isolate pure reaction products. Spectral data of the substituted resveratrol derivatives are in accordance with the literature [17,19].

Once formed, plant polyphenols are prone to oxidation by tyrosinases, present in the plants' cells [24,25,31]. In the present study, substituting the hydroxy group at the C-4′ (*para*) position of resveratrol blocked completely its enzymic oxidation by tyrosinases from two different sources, whether the substrate was a 4′ methoxide or 4′ glucoside. This is in agreement with the results of Fenoll et al. [40], who reported that oxidation of *m*-hydroxylated monophenols by tyrosinase is less efficient than that of the *p*-hydroxylated monophenols. Our results also agree with those of Espin and Wichers [24], who suggested that resveratrol would be oxidized preferentially by tyrosinase at the 4′ hydroxy group to produce 3,5,3′,4′-tetrahydroxystilbene.

Recently, 3,5-dihydroxy-4′-*O*-methoxystilbene was reported to be a good inhibitor of mushroom tyrosinase in the oxidation of L-tyrosine and L-DOPA [41,42]. The structure of 4-substituted benzaldehydes, competitive inhibitors of tyrosinase [43], resembles that of the 3,5-dihydroxy-4′-*O*-methoxystilbene. Together with our findings that this compound cannot be oxidized by tyrosinase, this may suggest that tyrosinase does interact with substituted resveratrol, but the interaction is futile. Other natural inhibitors of tyrosinase, having a substituted resorcinol skeleton [44,45], point to the possible role of resorcinol structure in this inhibition. As 4-substituted resveratrol also contains a resorcinol moiety, it cannot be deduced which of the above structures function in the inhibition. Substituted resorcinols were shown recently to inhibit melanin biosynthesis [44]. Thus the notion that beneficial polyphenols must be good electron donors or powerful antioxidants *in vitro* [21,46] is not necessarily accurate. Derivatives of resveratrol that contain non-oxidizable small groups at the 4′ position and a resorcinol skeleton may be naturally occurring inhibitors of the browning of fruits and vegetables, as well as of pigmentation and melanin formation in cancer cells [44]. It is well established that polyphenols and their glycosides are subjected to enzymic and microbial metabolism in the gastrointestinal tract and in the liver, resulting in the occurrence of glucoronidated, methylated and sulphated products in the circulating plasma [15]. These metabolites may act *in vivo* by influencing intracellular and redox-sensitive signalling pathways and gene expression, as discussed thoroughly in a recent review by Youdim et al. [5].

We determined the enzyme kinetics of the fungal and grape tyrosinases, using the resveratrol substituted at C-3. Both mushroom and grape tyrosinases behaved similarly, despite their genetic gap, suggesting that tyrosinases isolated from various sources have similar structural and functional characteristics, as observed previously [26]. Moreover, it suggests that resveratrol may serve as an excellent model substrate to study the enzymic oxidation of polyphenols by tyrosinases.

The V_{max} values did not differ largely with the substitution of the hydroxy group at C-3 by either a methoxy or glucosyl group. This re-emphasizes the requirement for the C-4′ hydroxy group for

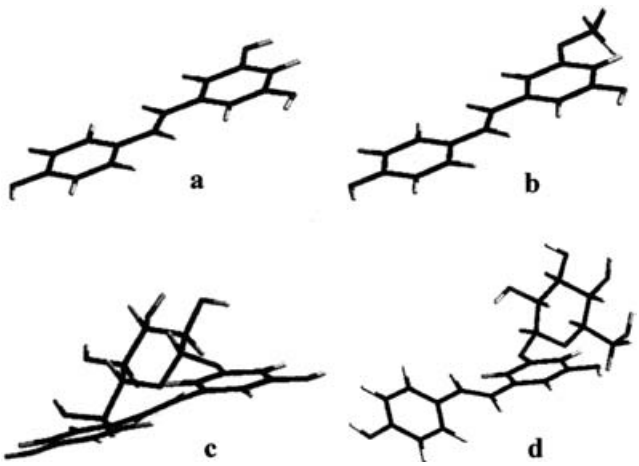

Figure 4 Calculated three-dimensional structures of resveratrol and its derivatives

Optimal geometries were calculated using the restricted Hartree–Fock AM1 Hamiltonian. All the calculations were performed with the Spartan 5.1 program package. Calculated three-dimensional optimal structures of (**a**) resveratrol, (**b**) 5,4′-dihydroxy-3-*O*-methoxystilbene, (**c**) piceid and (**d**) three-dimensional higher energy conformation of piceid.

enzymic oxidation. The 4′-hydroxy group of resveratrol is also the more reactive in scavenging free radicals, as was shown in several assays [18,19]. In plants, polyphenols are found predominantly as glycosides [22]. Interestingly, most of the polyphenol glycosides in nature (i.e. piceid, genistein and daidzein) are glycosylated at the less reactive positions, in terms of antioxidant capacity [13,18,20]. For instance, blocking the C-7 hydroxy group of genistein or daidzein did not affect their antioxidant capacity, suggesting that the presence of a hydroxy group at position C-7 has a negligible effect on the antioxidant activities of these compounds. On the other hand, substituting the C-4′ hydroxy group on the flavonoid B-ring was demonstrated to have a dramatic effect on its ability to inhibit lipid peroxidation [20].

Similarity between the V_{max} values does suggest that once the enzyme has formed a complex with the substrate, oxidation may occur. Interestingly, methylation or glycosylation at the C-3 position did have a large influence on the affinity of the enzyme for its substrate. The oxygen atom at the C-3 position was estimated to be approx. 11 Å (1 Å = 0.1 nm) distant from the oxygen at the C-4′ position (which interacts with the active site of the enzyme) and thus the influence of modifications at C-3 on this enzyme–substrate affinity is not clear.

Since the *m*-dihydroxyphenyl ring of resveratrol is not oxidized by the enzyme, it is not clear how the remote substitution on this ring lowers the affinity of the enzyme for its substrate. It may be suggested that the more hydrophilic nature of the glucose moiety leads to the binding of water molecules, thus inhibiting a correct introduction of the substrate, or that the bulkiness of the glucose moiety itself is the inhibiting feature. The three-dimensional structure of the active site may support these suggestions. Both tyrosinases exhibited higher affinity towards the C-3-methoxylated resveratrol, whereas introduction of a bulky, hydrophilic substitute at the same position (glucose) greatly reduced the affinity. The enzymes tyrosinase, catecholoxidase and haemocyanin all share a similar active site, although their physiological functions differ. It has been shown that catecholoxidases and haemocyanin contain a hydrophobic pocket with an aromatic amino acid at the gate of the active site [47]. The amino acid phenylalanine may repulse the glucosyl substitute and thus interfere with the binding of the substrate to the enzyme.

Knowledge of the three-dimensional structure of the substrate may shed more light on the enzyme–substrate interaction. According to semi-empirical quantum chemical calculations, resveratrol is a planar molecule (Figure 4a), containing *p*-monohydroxyphenyl and *m*-dihydroxyphenyl moieties *trans*-bonded to the ethane fragment (Figure 1). Interestingly, the same considerations show that whereas 5,4′-dihydroxy-3-*O*-methoxystilbene is planar (Figure 4b), like resveratrol, piceid is twisted along the ethane bridge (Figure 4c). This conformational change may be one of the factors contributing to the increased resistance towards enzymic oxidation. It may also be suggested that, based on this three-dimensional conformation, the glucose residue itself poses a steric hindrance that inhibits the fertile entrance of the monohydroxylated ring into the active site. This may lead to complete inhibition of enzymic oxidation of piceid. However, there is another stable conformation of piceid (Figure 4d), which is 1.54 kcal/mol higher in energy than the previous one and keeps the double bond twisted. The glucoside moiety in this conformation is far from the monohydroxylated ring, thus allowing some oxidation to take place.

Several recent studies have evaluated the kinetic parameters of tyrosinase using two common substrates. The k_{cat} values found in the present study for resveratrol and its derivatives were all higher than that of tyrosine but lower than that of DOPA [48,49]. The $t_{1/2}$ of resveratrol, measured here, in a dilute enzyme solution, is three times lower than that of piceid. These differences will only be of biological significance if they result in an extension of the lifespan of newly synthesized polyphenols in plants that contain substrates, described previously [25], and potential inhibitors of tyrosinase [44,50]. It was shown earlier that resveratrol is oxidized quickly in fresh grape juice and that this oxidation may be inhibited by the addition of inhibitors such as SO_2 [25]. We used grape juice (major dietary source of many polyphenols and raw material for wine production) with high tyrosinase activity (results not shown) as a model plant extract. It was found that even in this crude grape juice both resveratrol and piceid were good substrates for tyrosinase. Still, $t_{\frac{1}{2}}$ of piceid was found to be 5.5 times higher than that of resveratrol at 20 °C, as in the dilute reaction mixture, and four times higher at 4 °C (Table 4). Ascorbic acid, another plant antioxidant, had no effect on this enzymic oxidation (results not shown).

It is believed generally that the glycosylation of polyphenols in plants serves to increase their solubility [22]. It has already been shown that piceid retains biological activities of resveratrol [51] and that the 4′-hydroxy group (and not the 3′ one) is the most important functional group in resveratrol in terms of its antioxidant activity [18,19]. We show in the present study that the 3-glucoside derivative is more resistant to enzymic oxidation and, thus, more stable in plant cells. It is suggested that glycosylation of resveratrol, and possibly other polyphenols, at their less reactive groups has evolved in plants to protect these vital molecules from deleterious oxidation by the plants' own PPOs, while maintaining their beneficial antioxidant activities and increasing their solubility in the cell cytoplasm.

We are grateful to Professor Orna Almog for her helpful discussion on protein structure. This work was supported in part by a scholarship to G.R.-S. from the Constantiner Foundation and in part by the Zigmond and Estel Lovenberg fund.

REFERENCES

1 Alluis, B. and Dangles, O. (2001) Quercetin (=2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-1-benzopyran-4-one) glycosides and sulfates: chemical synthesis, complexation, and antioxidant properties. Helv. Chim. Acta **84**, 1133–1156

2 Bravo, L. (1998) Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. Nutr. Rev. **56**, 317–333

3 Ursini, F. and Sevanian, A. (2002) Wine polyphenols and optimal nutrition. Ann. N. Y. Acad. Sci. **957**, 200–209
4 Fang, J. G., Lu, M., Chen, Z. H., Zhu, H. H., Li, Y., Yang, L., Wu, L. M. and Liu, Z. L. (2002) Antioxidant effects of resveratrol and its analogues against the free-radical-induced peroxidation of linoleic acid in micelles. Chemistry **8**, 4191–4198
5 Youdim, K. A., Spencer, J. P., Schroeter, H. and Rice-Evans, C. (2002) Dietary flavonoids as potential neuroprotectants. Biol. Chem. **383**, 503–519
6 Spencer, J. P., Schroeter, H., Kuhnle, G., Srai, S. K., Tyrrell, R. M., Hahn, U. and Rice-Evans, C. (2001) Epicatechin and its *in vivo* metabolite, 3′-*O*-methyl epicatechin, protect human fibroblasts from oxidative-stress-induced cell death involving caspase-3 activation. Biochem. J. **354**, 493–500
7 Spencer, J. P., Kuhnle, G. G., Williams, R. J. and Rice-Evans, C. (2003) Intracellular metabolism and bioactivity of quercetin and its *in vivo* metabolites. Biochem. J. **372**, 173–181
8 Sato, M., Maulik, N. and Das, D. K. (2002) Cardioprotection with alcohol: role of both alcohol and polyphenolic antioxidants. Ann. N. Y. Acad. Sci. **957**, 122–135
9 Zou, J. G., Wang, Z. R., Houang, Y. Z., Cao, K. J. and Wu, J. M. (2003) Effect of red wine and wine polyphenol resveratrol on endothelial function in hypercholesterolemic rabbits. Int. J. Mol. Med. **11**, 317–320
10 Schroeter, H., Spencer, J. P., Rice-Evans, C. and Williams, R. J. (2001) Flavonoids protect neurons from oxidized low-density-lipoprotein-induced apoptosis involving c-Jun N-terminal kinase (JNK), c-Jun and caspase-3. Biochem. J. **358**, 547–557
11 Jang, M. S., Cai, E. N., Udeani, G. O., Slowing, K. V., Thomas, C. F., Beecher, C. W. W., Fong, H. H. S., Farnsworth, N. R., Kinghorn, A. D., Mehta, R. G. et al. (1997) Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science **275**, 218–220
12 Price, K. R. and Fenwick, G. R. (1985) Naturally occurring oestrogens in foods – a review. Food Addit. Contam. **2**, 73–106
13 Goldberg, D. M., Ng, E., Karumanchiri, A., Diamandis, E. P. and Soleas, G. J. (1996) Resveratrol glucosides are important components of commercial wines. Am. J. Enol. Vitic. **47**, 415–420
14 Langcake, P., Cornford, C. A. and Pryce, R. J. (1979) Identification of pterostilbene as a phytoalexin from *Vitis vinifera* grape leaves tested on *Cladisporium cucumerinum* and *Plasmopara viticola*, fungi. Phytochemistry **18**, 1025–1027
15 Spencer, J. P., Schroeter, H., Rechner, A. R. and Rice-Evans, C. (2001) Bioavailability of flavan-3-ols and procyanidins: gastrointestinal tract influences and their relevance to bioactive forms *in vivo*. Antioxidants Redox Signalling **3**, 1023–1039
16 Kuhnle, G., Spencer, J. P. E., Chowrimootoo, G., Schroeter, H., Debnam, E. S., Srai, S. K., Rice-Evans, C. and Hahn, U. (2000) Resveratrol is absorbed in the small intestine as resveratrol glucuronide. Biochem. Biophys. Res. Commun. **272**, 212–217
17 Orsini, F., Pelizzoni, F., Bellini, B. and Miglierini, G. (1997) Synthesis of biologically active polyphenolic glycosides (combretastatin and resveratrol series). Carbohydr. Res. **301**, 95–109
18 Stojanovic, S. and Brede, O. (2002) Elementary reaction of the antioxidant action of *trans*-stilbene derivatives: resveratrol, pinosylvin and 4-hydroxystilbene. Phys. Chem. Chem. Phys. **4**, 757–764
19 Stivala, L. A., Savio, M., Carafoli, F., Perucca, P., Bianchi, L., Maga, G., Forti, L., Pagnoni, U. M., Albini, A., Prosperi, E. et al. (2001) Specific structural determinants are responsible for the antioxidant activity and the cell cycle effects of resveratrol. J. Biol. Chem. **276**, 22586–22594
20 Arora, A., Nair, M. G. and Strasburg, G. M. (1998) Antioxidant activities of isoflavones and their biological metabolites in a liposomal system. Arch. Biochem. Biophys. **356**, 133–141
21 Pannala, A. S., Chan, T. S., O'Brien, P. J. and Rice-Evans, C. A. (2001) Flavonoid B-ring chemistry and antioxidant activity: fast reaction kinetics. Biochem. Biophys. Res. Commun. **282**, 1161–1168
22 Vickery, M. L. and Vickery, B. (1981) Secondary Plant Metabolism, vol. 1, pp. 20–55, The Macmillan Press Ltd, Hong Kong
23 Gee, J. M., DuPont, M. S., Rhodes, M. J. C. and Johnson, I. T. (1998) Quercetin glucosides interact with the intestinal glucose transport pathway. Free Radical Biol. Med. **25**, 19–25
24 Espin, J. C. and Wichers, H. J. (2000) Study of the oxidation of resveratrol catalyzed by polyphenol oxidase. Effect of polyphenol oxidase, laccase and peroxidase on the antiradical capacity of resveratrol. J. Food Biochem. **24**, 225–250
25 Gilly, R., Mara, D., Oded, S. and Zohar, K. (2001) Resveratrol and a novel tyrosinase in Carignan grape juice. J. Agric. Food Chem. **49**, 1479–1485
26 Robb, D. A. (1984) Tyrosinase. In Copper Proteins and Copper Enzymes, vol. 2 (Lontie, R., ed.), pp. 207–241, CRC Press, Boca Raton, FL
27 Mayer, A. and Harel, E. (1979) Polyphenol oxidases in plants. Phytochemistry **18**, 193–215
28 Sanchez-Ferrer, A., Rodriguez-Lopez, J. N., Garcia-Canovas, F. and Garcia-Carmona, F. (1995) Tyrosinase: a comprehensive review of its mechanism. Biochim. Biophys. Acta **1247**, 1–11
29 Solomon, E. I., Sundaram, U. M. and Machonkin, T. E. (1996) Multicopper oxidases and oxygenases. Chem. Rev. **96**, 2563–2606
30 Rodriguez-Lopez, J. N., Tudela, J., Varon, R., Garcia-Carmona, F. and Garcia-Canovas, F. (1992) Analysis of a kinetic model for melanin biosynthesis pathway. J. Biol. Chem. **267**, 3801–3810
31 Fan, X. and Mattheis, J. P. (2001) Inhibition of oxidative and antioxidative enzymes by *trans*-resveratrol. J. Food Sci. **66**, 200–203
32 Fuhrman, B., Lavy, A. and Aviram, M. (1995) Consumption of red wine with meals reduces the susceptibility of human plasma and low-density lipoprotein to lipid peroxidation. Am. J. Clin. Nutr. **61**, 549–554
33 Soleas, G. J., Diamandis, E. P. and Goldberg, D. M. (2001) The world of resveratrol. Adv. Exp. Med. Biol. **492**, 159–182
34 Cardona, M. L., Fernandez, I., Garcia, B. M. and Pedro, R. J. (1986) Synthesis of natural polyhydroxystilbene. Tetrahedron **42**, 2725–2730
35 Koenigs, W. and Knorr, E. (1901) Ueber einige derivate des traubenzuckers und der galactose. Chem. Ber. **34**, 957–981
36 Privat, C., Telo, J. P., Bernardes, G. V., Vieira, A., Souchard, J. P. and Nepveu, F. (2002) Antioxidant properties of *trans*-ε-viniferin as compared to stilbene derivatives in aqueous and nonaqueous media. J. Agric. Food Chem. **50**, 1213–1217
37 Cash, J. N., Sistrunk, W. A. and Stutte, C. A. (1976) Characteristics of concord grape polyphenoloxidase involved in juice color loss. J. Food Sci. **41**, 1398–1402
38 Ponticelli, F., Trendafilova, A., Valoti, M., Saponara, S. and Sgaragli, G. (2001) Synthesis and antiperoxidant activity of new phenolic *O*-glycosides. Carbohydr Res. **330**, 459–468
39 Spencer, J. P., Schroeter, H., Crossthwaithe, A. J., Kuhnle, G., Williams, R. J. and Rice-Evans, C. (2001) Contrasting influences of glucuronidation and *O*-methylation of epicatechin on hydrogen peroxide-induced cell death in neurons and fibroblasts. Free Radical Biol. Med. **31**, 1139–1146
40 Fenoll, L. G., Rodriguez-Lopez, J. N., Varon, R., Garcia-Ruiz, P. A., Garcia-Canovas, F. and Tudela, J. (2000) Action mechanism of tyrosinase on *meta*- and *para*-hydroxylated monophenols. Biol. Chem. **381**, 313–320
41 Shin, N. H., Ryu, S. Y., Choi, E. J., Kang, S. H., Chang, I. M., Min, K. R. and Kim, Y. (1998) Oxyresveratrol as the potent inhibitor on dopa oxidase activity of mushroom tyrosinase. Biochem. Biophys. Res. Commun. **243**, 801–803
42 Kim, Y. M., Yun, J., Lee, C. K., Lee, H., Min, K. R. and Kim, Y. (2002) Oxyresveratrol and hydroxystilbene compounds. Inhibitory effect on tyrosinase and mechanism of action. J. Biol. Chem. **277**, 16340–16344
43 Jimenez, M., Chazarra, S., Escribano, J., Cabanes, J. and Garcia-Carmona, F. (2001) Competitive inhibition of mushroom tyrosinase by 4-substituted benzaldehydes. J. Agric. Food Chem. **49**, 4060–4063
44 Nerya, O., Vaya, J., Musa, R., Izrael, S., Ben-Arie, R. and Tamir, S. (2003) Glabrene and isoliquiritigenin as tyrosinase inhibitors from licorice roots. J. Agric. Food Chem. **51**, 1201–1207
45 Shimizu, K., Kondo, R. and Sakai, K. (2000) Inhibition of tyrosinase by flavonoids, stilbenes and related 4-substituted resorcinols: structure–activity investigations. Planta Med. **66**, 11–15
46 Rice-Evans, C. A., Miller, N. J. and Paganga, G. (1996) Structure–antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biol. Med. **20**, 933–956
47 Decker, H. and Tuczek, F. (2000) Tyrosinase/catecholoxidase activity of hemocyanins: structural basis and molecular mechanism. Trends Biochem. Sci. **25**, 392–397
48 Espin, J. C., Garcia-Ruiz, P. A., Tudela, J. and Garcia-Canovas, F. (1998) Study of stereospecificity in mushroom tyrosinase. Biochem. J. **331**, 547–551
49 Fenoll, L. G., Rodriguez-Lopez, J. N., Varon, R., Garcia-Ruiz, P. A., Garcia-Canovas, F. and Tudela, J. (2000) Kinetic study of the oxidation of 3-hydroxyanisole catalysed by tyrosinase. Biophys. Chem. **84**, 65–76
50 Lee, H. S. (2002) Tyrosinase inhibitors of Pulsatilla cernua root-derived materials. J. Agric. Food Chem. **50**, 1400–1403
51 Kimura, Y. and Okuda, H. (2000) Effects of naturally occurring stilbene glucosides from medicinal plants and wine, on tumour growth and lung metastasis in Lewis lung carcinoma-bearing mice. J. Pharm. Pharmacol. **52**, 1287–1295

Received 20 January 2003/14 April 2003; accepted 16 April 2003
Published as BJ Immediate Publication 16 April 2003, DOI 10.1042/BJ20030141